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(54) Title: USE OF CROSSLINKED CRYSTALS AS A NOVEL FORM OF ENZYME IMMOBILIZATION (57) Abstract A method of immobilizing an enzyme by forming crystals of the enzyme and, generally, also cross-linking the resulting crystals through the use of a bifunctional reagent; crosslinked, immobilized enzyme crystals (CLECs) made by this method; lyophilization of CLECs made by this method; lyophilized crosslinked immobilized CLECs and a method of making a desired product by means of a reaction catalyzed by a CLEC or set of CLECs.		

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USE OF CROSSLINKED CRYSTALS AS A NOVEL FORM OF
ENZYME IMMOBILIZATION

Related Application

This application is a Continuation-in-Part of
5 U.S.S.N. 07/562,280 filed August 3, 1990 and entitled
"Use of Crosslinked Crystals as a Novel Form of Enzyme
Immobilization". The teachings of U.S.S.N. 07/562,280
are incorporated herein by reference.

Background of the Invention

10 Enzymes are used as industrial catalysts for the
large and laboratory scale economical production of
fine and specialty chemicals (Jones, J.B., Tetrahedron
42: 3351-3403 (1986)), for the production of foodstuffs
(Zaks et. al., Trends in Biotechnology 6: 272-275
15 (1988)), and as tools for the synthesis of organic
compounds (Wong, C.-H., Science 244: 1145-1152 (1989);
CHEMTRACTS-Org. Chem. 3: 91-111 (1990); Klibanov,
A.M., Acc. Chem. Res. 23: 114-120 (1990)).

Enzyme-based manufacturing can significantly
20 reduce the environmental pollution burden implicit in
the large scale manufacturing of otherwise unusable
chemical intermediates, as shown in the large scale
production of acrylamide using the enzyme, nitrile
hydratase (Nagasawa, T. and Yamada, H., Trends in
25 Biotechnology 7: 153-158 (1989)).

Enzymes are also used in biosensor applications to
detect various substances of clinical, industrial and
other interest (Hall, E., "Biosensors", Open University
Press (1990)). In the clinical area, enzymes may be
30 used in extracorporeal therapy, such as hemodialysis
and hemofiltration, where the enzymes selectively
remove waste and toxic materials from blood (Klein, M.

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and Langer, R., Trends in Biotechnology 4: 179-135 (1986)). Enzymes are used in these areas because they function efficiently as catalysts for a broad range of reaction types, at modest temperatures, and with

5 substrate specificity and stereoselectivity.

Nonetheless, there are disadvantages associated with the use of soluble enzyme catalysts which have limited their use in industrial and laboratory chemical processes (Akiyama et. al., CHEMTECH 627-634 (1988)).

10 Enzymes are expensive and relatively unstable compared to most industrial and laboratory catalysts, even when they are used in aqueous media where enzymes normally function. Many of the more economically interesting chemical reactions carried out in common
15 practice are incompatible with aqueous media, where, for example, substrates and products are often insoluble or unstable, and where hydrolysis can compete significantly. In addition, the recovery of soluble enzyme catalyst from product and unreacted substrate in
20 the feedstock often requires the application of complicated and expensive separation technology. Finally, enzymes are difficult to store in a manner that retains their activity and functional integrity, for commercially reasonable periods of time (months to years)
25 without having to resort to refrigeration (4°C to -80°C to liquid N₂ temperatures), or to maintenance in aqueous solvents of suitable ionic strength, pH, etc.

Enzyme immobilization methods have, in many instances, circumvented these disadvantages.

30 Immobilization can improve the stability of enzyme

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catalysts and protect their functional integrity in the harsh solvent environments and extreme temperatures characteristic of industrial and laboratory chemical processes (Hartmeier, W., Trends in Biotechnology 3: 149-153 (1985)). Continuous flow processes may be operated with immobilized enzyme particles in columns, for example, where the soluble feedstock passes over the particles and is gradually converted into product.

As used herein, the term enzyme immobilization refers to the insolubilization of enzyme catalyst by attachment to, encapsulation of, or by aggregation into macroscopic (10^{-1} mm) particles.

A number of useful reviews of enzyme immobilization methods have appeared in the literature (Maugh, T.H., Science 223: 474-476 (1984); Tramper, J., Trends in Biotechnology 3: 45-50 (1985)). Maugh describes five general approaches to the immobilization of enzymes. These include: adsorption on solid supports (such as ion-exchange resins); covalent attachments to supports (such as ion-exchange resins, porous ceramics or glass beads); entrapment in polymeric gels; encapsulation; and the precipitation of soluble proteins by cross-linking them with bifunctional reagents in a random and undefined manner. In addition, one can immobilize whole cells (usually dead and made permeable) which have expressed the desired enzyme activity at high levels (e.g., Nagasawa, T. and Yamada, H., Trends in Biotechnology 7: 153-158 (1989)).

Each of these immobilization procedures has its own advantages and limitations and none can be

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considered optimal or dominating. In most of them, the enzyme catalyst ultimately represents only a small fraction of the total volume of material present in the chemical reactor. As such, the bulk of the immobilized medium is made up of inert, but often costly carrier material. In all of them, the immobilizing interactions of the enzyme catalyst molecules with each other and/or with the carrier material tend to be random and undefined. As a result, although these interactions confer some enhanced stability to the enzyme catalyst molecules, their relative non-specificity and irregularity makes that stabilization sub-optimal and irregular. In most cases, access to the active site of the enzyme catalyst remains ill-defined. In addition, the immobilization methods described above fail to deal with problems associated with storage and refrigeration. Nor can conventionally immobilized enzymes generally be manipulated, as in being exchanged into one or another solvent of choice, without risk to the structural and functional integrity of the enzyme. In practical terms, except for the attached tether to the carrier particle, conventionally immobilized enzymes bear close resemblance to soluble enzymes, and share with them a susceptibility to denaturation and loss of function in harsh environments. In general, immobilization methods lead to a reduction of observed enzyme-catalyzed reaction rates relative to those obtained in solution. This is mostly a consequence of the limits of inward diffusion of substrate and outward

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diffusion of product within the immobilized enzyme particle (Quioco, F.A., and Richards, F.M., Biochemistry 5: 4062-4076 (1967)). The necessary presence of inert carrier in the immobilized enzyme particles increases the mean free path between the solvent exterior of the immobilized enzyme particle and the active site of the enzyme catalyst and thus exacerbates these diffusion problems. When dealing with immobilized cells, the diffusion problem is particularly severe, even if cell walls and membranes are made permeable to substrate and product in some way. One would further be concerned with the multitude of contaminating enzymatic activities, metabolites, and toxins contained in cells, and with the stability of cells in harsh solvents or extreme temperature operating environments. An improved immobilization technique which avoids the limitations of the presently available methods would be helpful in promoting the use of enzymes as industrial catalysts, particularly if it were shown to be useful on a large scale (Daniels, M.J., Methods in Enzymology 136: 371-379 (1987)).

Summary of the Invention

The present invention relates to a method of immobilizing an enzyme by forming crystals of the enzyme and, generally, also cross linking the resulting crystals through use of a bifunctional reagent; cross-linked immobilized enzyme crystals (referred to as CLECs or CLIECs) made by this method; the lyophilization of CLECs as a means of improving the

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storage, handling, and manipulation properties of immobilized enzymes and a method of making a desired product by means of a reaction catalyzed by a CLEC or a set of CLECs.

5 In the method of the present invention, small (10⁻⁴mm) protein crystals are grown from aqueous solutions, or aqueous solutions containing organic solvents, in which the enzyme catalyst is structurally and functionally stable. In a preferred embodiment,
10 crystals are then cross-linked with a bifunctional reagent, such as glutaraldehyde. This cross-linking results in the stabilization of the crystal lattice contacts between the individual enzyme catalyst molecules constituting the crystal. As a result of
15 this added stabilization, the cross-linked immobilized enzyme crystals can function at elevated temperatures, extremes of pH and in harsh aqueous, organic, or near-anhydrous media, including mixtures of these. That is, a CLEC of the present invention can function
20 in environments incompatible with the functional integrity of the corresponding uncrystallized, uncrosslinked, native enzyme or conventionally immobilized enzyme catalysts.

In addition, CLECs made by this method can be
25 subjected to lyophilization, producing a lyophilized CLEC which can be stored in this lyophilized form at non-refrigerated (room) temperatures for extended periods of time, and which can be easily reconstituted in aqueous, organic, or mixed aqueous-organic solvents
30 of choice, without the formation of amorphous

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suspensions and with minimal risk of denaturation.

The present invention also relates to CLECs produced by the present method and to their use in laboratory and large scale industrial production of
5 selected materials, such as chiral organic molecules, peptides, carbohydrates, lipids, or other chemical species. Presently, these are typically prepared by conventional chemical methods, which may require harsh conditions (e.g. aqueous, organic or near-anhydrous
10 solvents, mixed aqueous/organic solvents or elevated temperatures) that are incompatible with the functional integrity of uncrystallized, uncrosslinked, native enzyme catalyst. Other macromolecules with catalytic activity can also be incorporated into the proposed
15 CLEC technology. These might include catalytic antibodies (Lerner, R.A., Benkovic, S.J., and Schultz, P.G., Science 252:659-667 (1991)) and catalytic polynucleotides (Cech, T.R., Cell 64:667-669 (1991); Celander, D.W., and Cech, T.R. Science, 251:401-407
20 (1991)).

The present invention also relates to a method of making a selected product by means of a reaction catalyzed by a CLEC of the present invention.

In an example of the method and practice of the
25 present invention, the enzyme thermolysin, a zinc metalloprotease, was used to synthesize a chiral precursor of the dipeptidyl artificial sweetener, aspartame. The enzyme thermolysin was crystallized from a starting aqueous solution of 45% dimethyl
30 sulfoxide, and 55% 1.4M calcium acetate, 0.05M sodium

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cacodylate, pH 6.5. The resulting crystals were cross-linked with glutaraldehyde to form a thermolysin CLEC. The thermolysin CLEC was then transferred from the aqueous crystallization solution in which it was made, into a solution of ethyl acetate containing the substrates, N-(benzyloxycarbonyl)-L-aspartic acid (Z-L-Asp) and L-phenylalanine methyl ester (L-Phe-OMe). The thermolysin CLEC was then used to catalyze a condensation reaction of the two substrates to synthesize N-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-L-Asp-L-Phe-OMe), which is the dipeptidyl precursor of the artificial sweetener aspartame. Using any one of many known techniques (see, e.g. Lindeberg, G., J. Chem Ed. 64: 1062-1064 (1987)) the L-aspartic acid in the synthesized dipeptidyl precursor can be deprotected by the removal of the benzyloxycarbonyl (Z-) group to produce aspartame (L-Asp-L-Phe-OMe).

In a second example of the method and practice of the present invention, the enzyme thermolysin was used to produce thermolysin CLECs. The activity and stability of thermolysin CLECs were compared to that of soluble thermolysin under optimum conditions and conditions of extreme pH and temperature, following incubation in the presence of organic solvents and following incubation in the presence of exogenous protease.

The enzyme thermolysin was crystallized from a solution of 1.2M calcium acetate and 30% dimethyl sulfoxide pH 8.0. The resulting crystals were

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crosslinked with glutaraldehyde at a concentration of 12.5% to form a thermolysin CLEC. The thermolysin CLEC was then lyophilized by a standard procedure (Cooper, T.G., The Tools of Biochemistry, pp. 379-380 (John Wiley and Sons, NY (1977)) to form a lyophilized enzyme CLEC of thermolysin. This lyophilized CLEC was then transformed directly into the different aqueous, organic, and mixed aqueous/organic solvents of choice without an intervening solvent exchange procedure, without formation of amorphous suspensions, and with minimal risk of denaturation. These solvents included acetonitrile, dioxane, acetone, and tetrahydrofuran, but not to the exclusion of others. Following incubation, activity was assayed spectrophotometrically by cleavage of the dipeptide substrate FAGLA (furylacryloyl-glycyl-L-leucine amide).

In a third example of the method and practice of the present invention, the enzyme elastase (porcine pancreatic) was crystallized from an aqueous solution of 5.5 mg/ml protein in 0.1 M sodium acetate at pH 5.0 at room temperature (Sawyer, L. et al., J. Mol. Biol. 118:137-208). The resulting crystals were crosslinked with glutaraldehyde at a concentration of 5% to form an elastase CLEC. The elastase CLEC was lyophilized as described in Example 2.

In a fourth example of the method and practice of the present invention, and as disclosed here, the enzyme esterase (porcine liver) was crystallized from an aqueous solution of 15 mg/ml protein in 0.25 M calcium acetate at pH 5.6 at room temperature. The

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resulting crystals were crosslinked with glutaraldehyde at a concentration of 12.5% to form an esterase CLEC. The esterase CLEC was lyophilized as described in Example 2.

5 In a fifth example of the method and practice of the present invention, and as disclosed here, the enzyme lipase (Geotrichum candidum) was crystallized from an aqueous solution of 20 mg/ml protein in 50 mM Tris at pH 7 at room temperature. The resulting
10 crystals were crosslinked with glutaraldehyde at a concentration of 12.5% to form a lipase CLEC. The lipase CLEC was lyophilized as described in Example 2.

In a sixth example of the method and practice of the present invention, the enzyme lysozyme (hen egg
15 white) was crystallized from an aqueous solution of 40 mg/ml protein in 40 mM sodium acetate buffer containing 5% sodium chloride at pH 7.4 at room temperature (Blake, C.C.F. et al., Nature, 196:1173 (1962)). The resulting crystals were crosslinked with glutaraldehyde
20 at a concentration of 20% to form a lysozyme CLEC. The lysozyme CLEC was lyophilized as described in Example 2.

In a seventh example of the method and practice of the present invention, the enzyme asparaginase
25 (Escherichia coli) was crystallized from an aqueous solution of 25 mg/ml protein in 50 mM sodium acetate and 33% ethanol at pH 5.0 at 4°C. The crystallization is a modification of the procedure described by Grabner et al. [U.S. Patent 3,664,926 (1972)]. As disclosed
30 here, the resulting crystals were crosslinked with

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glutaraldehyde at a concentration of 7.5% to form an asparaginase CLEC. The asparaginase CLEC was lyophilized as described in Example 2.

Other enzymes which can be immobilized in a similar manner and used to catalyze an appropriate reaction include luciferase and urease. Other enzymes, such as those listed in Tables 1-5, can also be crystallized and crosslinked using the present method, to produce a desired CLEC which can, in turn, be used to catalyze a reaction which results in production of a selected product or to catalyze a reaction which is an intermediate step (i.e. one in a series of reactions) in the production of a selected product. It is recognized that although crosslinking helps stabilize the majority of crystals, it is neither necessary nor desirable in all cases. Some crystalline enzymes retain functional and structural integrity in harsh environments even in the absence of crosslinking. Although in the preferred embodiment, the crystal is crosslinked, crosslinking is not always necessary to produce an enzyme crystal useful in the present method.

CLECs have several key characteristics that confer significant advantages over conventional enzyme immobilization methods presently in use. CLECs dispense with the need for a separate, inert support structure. Lack of an inert support will improve substrate and product diffusion properties within CLECs and provides enzyme concentrations within the crystal that are close to the theoretical packing limit for molecules of such size. High enzyme concentrations

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can lead to significant operational economies through the increased effective activity of a given volume of catalyst, reduction in substrate contact time with enzyme and overall reduction in plant size and capital costs (Daniels, M.J., Methods in Enzymol. 136: 371-379 (1987)). The uniformity across crystal volume and enhanced stability of the constituent enzyme in CLECs creates novel opportunities for the use of enzyme catalysis in harsh conditions, such as elevated temperature, and aqueous, organic or near-anhydrous solvents, as well as mixtures of these. In addition, the restricted solvent access and regular protein environment implicit in a crystal lattice should lead to improved metal ion and co-factor retention for CLECs vs. conventional immobilized enzyme systems.

Brief Description of the Drawings

Figure 1 is a graphic representation of results of assessment of enzymatic activity of soluble and thermolysin CLEC.

Figure 2 is a graphic representation of results of a comparison of pH dependencies of thermolysin CLEC and soluble thermolysin.

Figure 3 is a graphic representation of measurement of the activity of soluble and crystalline thermolysin after incubation at 65°C.

Figure 4 is a graphic representation of results of assessment of resistance of soluble and thermolysin CLEC to exogenous proteolytic degradation.

Figure 5 is a graphic representation of results of

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the assessment of enzymatic activity for soluble elastase and the corresponding elastase CLEC.

Figure 6 is a graphic representation of the resistance of soluble elastase and the corresponding elastase CLEC to exogenous proteolytic degradation.

Figure 7 is a graphic representation of results of the assessment of enzymatic activity for soluble esterase and the corresponding esterase CLEC.

Figure 8 is a graphic representation of the resistance of soluble esterase and the corresponding esterase CLEC to exogenous proteolytic degradation.

Figure 9 is a graphic representation of results of the assessment of enzymatic activity for soluble lipase and the corresponding lipase CLEC.

Figure 10 is a graphic representation of results of the assessment of enzymatic activity for soluble lysozyme and the corresponding lysozyme CLEC.

Figure 11 is a graphic representation of results of the assessment of enzymatic activity for soluble asparaginase and the corresponding asparaginase CLEC.

Detailed Description of the Invention

A simple, general procedure that assures stability and function for a given enzyme or set of enzymes under conditions which are of interest to the synthetic chemist and which are too harsh for use with enzymes using presently available methods, would be very useful. Cross-linked immobilized enzyme crystals (referred to as CLECs or CLIECs) as described here can be used for this purpose. Stabilization of the crystal

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lattice and of the constituent enzyme catalysts in the crystal by the cross-linking reaction permits the use of CLECs in environments, including aqueous, organic or near-anhydrous solvents, mixtures of these solvents, extremes of pH and elevated temperatures, which are incompatible with enzyme function using presently available methods. In addition, the stabilization of the crystal lattice in CLECs makes possible the lyophilization of CLECs by standard methods.

10 Lyophilized CLECs can be stored for commercially attractive periods of time (months to years) in the absence of refrigeration, and facilitate the rapid and uncomplicated utilization of CLECs in industrial and laboratory scale processes by the simple addition of

15 solvents of choice, without need for intervening solvent exchange processes. CLECs are also highly resistant to digestion by exogenous proteases. The method of the present invention facilitates the use of versatile enzyme catalysts in mainstream industrial

20 chemical processes, as well as in the laboratory synthesis of novel compounds for research.

Although crosslinking contributes to the stability of a crystal enzyme, it is neither necessary nor desirable in all cases. Some crystallized enzymes

25 retain functional and structural integrity in harsh environments even in the absence of crosslinking. The preferred embodiment of the present method includes cross-linking of a crystal enzyme and is described in detail in the following sections. It is to be

30 understood, however, that crystallized enzymes which

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are not subsequently cross-linked can be used in some embodiments of the present invention.

The regular interactions between the constituent enzyme molecules in the crystal lattice of a CLEC
5 result in well defined pores of limited size leading to the enzyme molecules within the body of a CLEC. As a result, substrates larger than the available pore size will not penetrate the body of the CLEC particle.

As a consequence of the limited pore size, many
10 enzymatic reactions of commercial and academic interest involving substrates larger than the pore size of the CLECs would be beyond the scope of the present invention. This would include most reactions involving large polymers, such as proteins, polynucleotides,
15 polysaccharides, and other organic polymers, where the number of polymeric subunits would be such as to make the polymer larger than the crystal pore size in CLECs. In such instances, however, catalysis can still take place on the CLEC surface.

20 The present invention is a method of immobilizing a selected protein, particularly an enzyme, by crystallizing and crosslinking the protein, resulting in production of a crosslinked immobilized enzyme crystal (CLEC) which can be used to catalyze production
25 of a selected product, such as a peptide, carbohydrate, lipid or chiral organic molecule. The present invention further relates to such CLECs and to a method of making a selected product by means of a CLEC-catalyzed reaction or CLEC-catalyzed step in a
30 series of reactions. In one embodiment of the present

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invention, the dipeptidyl precursor of aspartame has been produced in a condensation reaction catalyzed by cross-linked immobilized thermolysin made by the present method. In another embodiment of this invention, the indicator substrate, FAGLA, has been cleaved to produce a colorimetric product, whose presence is indicative of enzyme activity in a thermolysin CLEC. FAGLA hydrolysis has been used as a model reaction to indicate the robustness of the thermolysin CLEC in a number of environments that would be normally incompatible with that enzyme's activity.

In other embodiments of this invention, the enzymes elastase, esterase, lipase, asparaginase, and lysozyme have been used to cleave various indicated substances, such as p-nitrophenyl acetate (esterase and lipase), succinyl- (ala)3-p-nitroanilide (elastase), 4-methylumbelliferyl N-acetyl-chitrioside (lysozyme) and NADH (asparaginase).

By the method of this invention, one of ordinary skill in the art can adapt a protocol for making a desired product by means of a reaction catalyzed by an immobilized enzyme. The enzyme of interest, when crystallized from an appropriate solution, can be cross-linked with glutaraldehyde or other suitable bifunctional reagent in the crystallization solution to produce a CLEC of that enzyme. Subsequently, the CLEC of the enzyme of choice can be lyophilized as described in Example 2.

There are several advantages which the use of a

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CLEC offers over presently-available enzyme-catalyzed methods. For example, the cross-linked crystal matrix in a CLEC provides its own support. Expensive carrier beads, glasses, gels, or films are not required in
5 order to tie down the enzyme catalyst, as they are in presently-available immobilization methods. As a result, the concentration of enzyme in a CLEC is close to the theoretical packing limit that can be achieved for molecules of a given size, greatly exceeding
10 densities achievable even in concentrated solutions. The entire CLEC consists of active enzyme (and not inactive carrier), and thus, the diffusion-related reduction of enzyme reaction rates usually observed with conventionally immobilized enzymes relative to
15 enzymes in solution should be minimized, since the mean free path for substrate and product between active enzyme and free solvent will be greatly shortened for CLECs (compared to a conventional immobilized enzyme carrier particles). These high protein densities
20 will be particularly useful in biosensor, analytical and other applications requiring large amounts of protein in small volumes. In industrial processes, the superior performance and compactness of CLECs results in significant operating economies, by
25 increasing the effective activity of a given volume of catalyst, thereby allowing reductions in plant size, as well as capital costs (Daniels, M.J., Methods in Enzymol. 136: 371-379 (1987)). CLECs are relatively monodisperse, with a macroscopic size and shape
30 reflecting natural crystal growth characteristics of

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the individual enzyme catalysts. Replacement of existing carrier-immobilized enzyme media with CLECs should not be difficult, since both systems are comparable in size and shape, and both can be similarly recovered from feedstock by any number of simple methods, including basic economical operations such as filtration, centrifugation, decantation of solvent, and others.

In addition, the use of lyophilized CLECs permits routine handling and storage of these materials prior to use (dry storage at room temperature without refrigeration, for extended periods of time). Lyophilized CLECs also allow for routine formulation by direct addition of solvents and substrates of interest, without lengthy solvent exchange processes, or the formation of amorphous suspensions. The lyophilized CLEC form extends the general utility of the enzymes as catalysts to a broader spectrum of enzymes and functional conditions.

A second advantage of a CLEC is that cross-linking of the crystallized enzyme stabilizes and strengthens the crystal lattice and the constituent enzyme molecules, both mechanically and chemically. As a result, a CLEC may be the only means of achieving significant concentrations of active enzyme catalyst in harsh aqueous, organic, near-anhydrous solvents, or in aqueous-organic solvent mixtures. The use of enzymes as catalysts in organic syntheses has been hampered by their tendency to denature in the presence of non-aqueous solvents, and particularly, in mixtures of

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aqueous and non-aqueous solvents (Klibanov, A.M., Trends in Biochemical Sciences, 14:141-144 (1989)). In CLECs, the restriction of conformational mobility that leads to stability is provided by the inter- molecular contacts and cross-links between the constituent enzyme molecules making up the crystal lattice, rather than by the near-absence of water in the medium. As a result, intermediate water concentrations can be tolerated by enzymes when formulated as CLECs, as has previously not been possible (see Table 12). In commercial applications, aqueous-organic solvent mixtures allow manipulation of product formation by taking advantage of relative solubilities of products and substrates. Even in aqueous media, enzyme catalysts, immobilized or soluble, are subject to mechanical forces within a chemical reactor that can lead to denaturation and a shortened half-life. The chemical cross-links within the CLEC provide the necessary mechanical strength (Quioco and Richards, Proc. Natl. Acad. Sci. (USA) 52: 833-839 (1964)) that results in increased reactor life for the enzyme catalyst.

A third advantage of a CLEC is that as a result of its crystalline nature, a CLEC can achieve uniformity across the entire cross-linked crystal volume. Crystalline enzymes as described herein are grown and cross-linked in an aqueous environment and, therefore, the arrangement of molecules within the crystal lattice remains uniform and regular. This uniformity is maintained by the intermolecular contacts and chemical cross-links between the enzyme molecules constituting

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the crystal lattice, even when exchanged into other aqueous, organic or near-anhydrous media, or mixed aqueous/organic solvents. In all of these solvents, the enzyme molecules maintain a uniform distance from each other, forming well-defined stable pores within the CLECs that facilitate access of substrate to the enzyme catalysts, as well as removal of product. Uniformity of enzyme activity is critical in industrial, medical and analytical applications where reproducibility and consistency are paramount.

A fourth advantage of using a CLEC is that it should exhibit an increased operational and storage half-life. Lattice interactions, even in the absence of cross-linking, are known to stabilize proteins, due in part to restrictions of the conformational degrees of freedom needed for protein denaturation. In CLECs, the lattice interactions, when fixed by chemical cross-links, are particularly important in preventing denaturation, especially in mixtures of aqueous and non-aqueous solvents (Klibanov, A.M., Trends in Biochemical Sciences 14: 141-144 (1989)). Enzymes that have been in the crystalline state for months or years routinely retain a high percentage of their catalytic activity. Cross-linked immobilized enzyme crystals stored in anhydrous solvents will be even further protected from microbial contamination and damage, which is a serious problem in storing large quantities of protein in a nutrient rich, aqueous environment. In the case of a lyophilized CLEC, the immobilized enzyme is stored in the absence of solvent. That, and

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the stabilization achieved by cross-linking allows for the storage in the absence of refrigeration for long periods of time.

A fifth advantage of using a CLEC is that it
5 should exhibit enhanced temperature stability as a consequence of the cross-links stabilizing the crystal lattice. Carrying out reactions at a higher temperature than that used with conventional methods would increase reaction rates for the chemical
10 reactions of interest, both thermodynamically, and by enhancing the diffusion rate into and out of the crystal lattice of CLECs. These combined effects would represent a major improvement in reaction efficiency, because they would maximize the productivity of a given
15 quantity of enzyme catalyst, which is generally the most expensive component of the reaction process (Daniels, M.J., Methods in Enzymol. 136: 371-379 (1987)). The temperature stability exhibited by CLECs is remarkable because most enzyme systems require mild
20 reaction conditions. CLECs would also be stabilized against denaturation by transient high temperatures during storage.

A final advantage of use of a CLEC is that pores of regular size and shape are created between
25 individual enzyme molecules in the underlying crystal lattice. This restricted solvent accessibility greatly enhances the metal ion or cofactor retention characteristics of CLEC as compared to conventionally immobilized enzymes and enzymes in solution. This
30 property of CLEC will permit the use of economically

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superior continuous-flow processes in situations (see e.g. Oyama et. al. Methods in Enzymol. 136 503-516 (1987)) where enzyme would otherwise be inactivated by metal ion or cofactor leaching. For example, in the thermolysin-mediated synthesis of the dipeptidyl aspartame precursor, Z-L-Asp-L-Phe-OMe, conventionally immobilized enzyme is known to lose catalytic activity in continuous-flow column processes, in part through the leaching of calcium ions essential for thermolysin activity. In practice, leaching of calcium ions has forced the use of less efficient batch processes (Nakanishi et. al., Biotechnology 3: 459-464 (1985)). Leaching occurs when calcium ion complexes are formed with substrate Z-L-Asp, in competition with the natural calcium binding sites on the surface of the enzyme, resulting in the loss of catalytic activity. The high density of enzyme, and the correspondingly limited volume accessible to solvent in the interstices of the CLECs, discourages the formation of the competing L-Asp-Ca⁺⁺ complexes responsible for metal ion leaching.

Preparation of CLECs - enzyme crystallization

In the method of the present invention, a cross-linked immobilized enzyme crystal (or CLEC) is prepared as follows:

Enzyme crystals are grown by the controlled precipitation of protein out of aqueous solution, or aqueous solution containing organic solvents. Conditions to be controlled include, for example, the

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rate of evaporation of solvent, the presence of appropriate co-solutes and buffers, and the pH and temperature. A comprehensive review of the various factors affecting the crystallization of proteins has
5 been published by McPherson (Methods Enzymol. 114: 112 (1985)). In addition, both McPherson and Gilliland (J. Crystal Growth 90: 51-59 (1988)) have compiled comprehensive lists of all proteins and nucleic acids that have been reported as crystallized, as well as the
10 conditions that lead to their crystallization. A compendium of crystals and crystallization recipes, as well as a repository of coordinates of solved protein and nucleic acid crystal structures, is maintained by the Protein Data Bank (Bernstein et. al. J. Mol. Biol.
15 112: 535-542 (1977)) at the Brookhaven National Laboratory. Such references can be used to determine the conditions necessary for the crystallization of a given protein or enzyme previously crystallized, as a prelude to the formation of an appropriate CLEC, and
20 can guide the formulation of a crystallization strategy for proteins that have not. Alternatively, an intelligent trial and error search strategy (see eg., Carter, C.W. Jr. and Carter, C.W., J. Biol. Chem. 254: 12219-12223 (1979)) can, in most instances, produce
25 suitable crystallization conditions for most proteins, including, but not limited to, those discussed above, provided that an acceptable level of purity can be achieved for these. The level of purity required can vary widely from protein to protein. In the case of
30 lysozyme, for example, the enzyme has been crystallized

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directly from its unpurified source, the hen egg-white (Gilliland, G.L., J. Crystal Growth 90: 51-59 (1988)).

For use as CLECs in the method of this invention, the large single crystals which are needed for X-ray diffraction analysis are not required, and may, in fact, be undesirable because of diffusion problems related to crystal size. Microcrystalline showers (ie. crystals of order 10^{-1} mm in size/cross section) are suitable for CLECs and are often observed, though seldom reported in the X-ray crystallographic literature. Micro-crystals are very useful in the method of this invention to minimize problems with diffusion (see eg., Quijcho, F.A., and Richards, F.M., Biochemistry 5: 4062-4076 (1967)).

In general, crystals are produced by combining the protein to be crystallized with an appropriate aqueous solvent or aqueous solvent containing appropriate precipitating agents, such as salts or organics. The solvent is combined with the protein at a temperature determined experimentally to be appropriate for the induction of crystallization and acceptable for the maintenance of protein stability and activity. The solvent can optionally include co-solutes, such as divalent cations, co-factors or chaotropes, as well as buffer species to control pH. The need for co-solutes and their concentrations are determined experimentally to facilitate crystallization. In an industrial scale process, the controlled precipitation leading to crystallization can best be carried out by the simple combination of protein, precipitant, co-solutes, and

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optionally buffers in a batch process. Alternative laboratory crystallization methods, such as dialysis, or vapor diffusion can also be adapted. McPherson (Methods Enzymol. 114: 112 (1985)), and Gilliland (J. Crystal Growth 90: 51-59 (1988)) include a comprehensive list of suitable conditions in their reviews of the crystallization literature. Occasionally, incompatibility between the cross-linking reagent and the crystallization medium might require exchanging the crystals into a more suitable solvent system.

Many of the proteins for which crystallization conditions have already been described in the literature, have considerable potential as practical enzyme catalysts in industrial and laboratory chemical processes, and are directly subject to formulation as CLECs within the method of this invention. Table 1 is a sampling of enzymes that have already been crystallized. Note that the conditions reported in most of these references have been optimized for the growth of large, diffraction quality crystals, often at great effort. Some degree of adjustment of conditions for the smaller crystals used in making CLECS might be necessary in some cases.

Table 1

Enzyme	Microbial or biological source	References (including those cited therein)
• alcohol dehydrogenase	horse liver	Eklund <i>et al.</i> , <i>J.Mol.Biol.</i> 146: 561-587 (1981)
• alcohol oxidase	<i>Pichia pastoris</i>	Boys <i>et al.</i> , <i>J.Mol.Biol.</i> 208: 211-212 (1989) Tykarska <i>et al.</i> , <i>J.Protein Chem.</i> 9: 83-86 (1990)
• aldolase (fructose-bisphosphate)	rabbit muscle calf muscle human muscle <i>Drosophila melanogaster</i>	Eagles <i>et al.</i> , <i>J.Mol.Biol.</i> 45: 533-544 (1969) Heidmer <i>et al.</i> , <i>Science</i> 171: 677-680 (1971) Goryunov <i>et al.</i> , <i>Biofizika</i> 14: 1116-1117 (1969) Millar <i>et al.</i> , <i>Trans.Roy.Soc.Lond. B293</i> : 209-214 (1981) Brenner <i>et al.</i> , <i>J..Biol.Chem.</i> 257: 11747-11749 (1982)
• aldolase (PKDG)	<i>Pseudomonas putida</i>	Vandlen <i>et al.</i> , <i>J.Biol.Chem.</i> 248: 2251-2253 (1973)
• alkaline phosphatase	<i>Escherichia coli</i>	Sowadski <i>et al.</i> , <i>J.Mol.Biol.</i> 150: 245-272 (1981)
• asparaginase	<i>Erwinia carotova</i> <i>Escherichia coli</i> <i>Escherichia coli</i> <i>Proteus vulgaris</i>	North <i>et al.</i> , <i>Nature</i> 224: 594-595 (1969) Epp <i>et al.</i> , <i>Eur.J.Biochem.</i> 20: 432-437 (1971) Yonei <i>et al.</i> , <i>J.Mol.Biol.</i> 110: 179-186 (1977) Tetsuya <i>et al.</i> , <i>J.Biol.Chem.</i> 248: 7620-7621 (1972)
• carbonic anhydrase	human erythrocyte (C) human erythrocyte (B) bovine erythrocyte	Kannan <i>et al.</i> , <i>J.Mol.Biol.</i> 12: 740-760 (1965) Kannan <i>et al.</i> , <i>J.Mol.Biol.</i> 63: 601-604 (1972) Carlsson <i>et al.</i> , <i>J.Mol.Biol.</i> 80: 373-375 (1973)
• catalase	horse erythrocyte <i>Micrococcus luteus</i> <i>Penicillium vitale</i> bovine liver	Glauser <i>et al.</i> , <i>Acta Cryst.</i> 21: 175-177 (1966) Marie <i>et al.</i> , <i>J.Mol.Biol.</i> 129: 675-676 (1979) Vainshtein <i>et al.</i> , <i>Acta Cryst.</i> A37: C29 (1981) Eventoff <i>et al.</i> , <i>J.Mol.Biol.</i> 103: 799-801 (1976)
• creatine kinase	bovine heart rabbit muscle	Gilliland <i>et al.</i> , <i>J.Mol.Biol.</i> 170: 791-793 (1983) McPherson, <i>J.Mol.Biol.</i> 81: 83-86 (1973)
• glutaminase	<i>Actenobacter glutanimasificans</i> <i>Pseudomonas</i> 7A	Wlodawer <i>et al.</i> , <i>J.Mol.Biol.</i> 99: 295-299 (1975) Wlodawer <i>et al.</i> , <i>J.Mol.Biol.</i> 112: 515-519 (1977)

Table 1 continued:

Enzyme	Microbial or biological source	References (including those cited therein)
• glucose oxidase	<i>Aspergillus niger</i>	Kalisz <i>et al.</i> , <i>J.Mol.Biol.</i> 213: 207-209 (1990)
• β -lactamases	<i>Staphylococcus aureus</i> <i>Bacillus cereus</i>	Moult <i>et al.</i> , <i>Biochem J.</i> 225: 167-176 (1985) Sutton <i>et al.</i> , <i>Biochem J.</i> 248: 181-188 (1987)
• lactate dehydrogenase	porcine chicken dogfish <i>Bacillus stearothermophilus</i>	Hackert <i>et al.</i> , <i>J.Mol.Biol.</i> 78: 665-673 (1973) Pickles <i>et al.</i> , <i>J.Mol.Biol.</i> 9: 598-600 (1964) Adams <i>et al.</i> , <i>J.Mol.Biol.</i> 41: 159-188 (1969) Schar <i>et al.</i> , <i>J.Mol.Biol.</i> 154: 349-353 (1982)
• lipase	<i>Geotrichum candidum</i> horse pancreatic <i>Mucor meihei</i> human pancreatic	Hata <i>et al.</i> , <i>J.Biochem.</i> 86: 1821-1827 (1979) Lombardo <i>et al.</i> , <i>J.Mol.Biol.</i> 205: 259-261 (1989) Brady <i>et al.</i> , <i>Nature</i> 343: 767-770 (1990) Winkler <i>et al.</i> , <i>Nature</i> 343: 771-774 (1990)
• luciferase	Firefly	Green, A.A., <i>et al.</i> , <i>Biochem. Biophys. Acta.</i> 20: 170 (1956)
• luciferase	<i>Vibrio harveyi</i>	Swanson <i>et al.</i> , <i>J.Biol.Chem.</i> 260: 1287-1289 (1985)
• nitrile hydratase	<i>Brevibacterium R312</i> <i>P. chlororaphis B23</i>	Nagasawa <i>et al.</i> , <i>Biochem.Biophys.Res. Commun.</i> 139: 1305-1312 (1986) Nagasawa <i>et al.</i> , <i>Eur J.Biochem.</i> 162: 691-698 (1987)
• peroxidase	horseradish horseradish roots (Type E4) Japanese radish	Braithwaite <i>et al.</i> , <i>J.Mol.Biol.</i> 106: 229-230 (1976) Aibara <i>et al.</i> , <i>J.Biochem.</i> 90: 489-496 (1981) Morita, <i>Acta Cryst.</i> A28: S52 (1979)
• peroxidase (chloride)	<i>Caldaromyces fumago</i>	Rubin <i>et al.</i> , <i>J.Biol.Chem.</i> 257: 7768-7769 (1982)
• peroxidase (cytochrome)	<i>Sarchomyces cerevisae</i>	Poulos <i>et al.</i> , <i>J.Biol.Chem.</i> 253: 3730-3735 (1978)
• peroxidase (glutathione)	bovine erythrocyte	Ladenstein <i>et al.</i> , <i>J.Mol.Biol.</i> 104: 877-882 (1979)
• subtilisin	<i>Bacillus subtilis</i> (Novo) <i>Bacillus amyloliquefaciens</i> (BPN) <i>Bacillus subtilis</i> (Carlsberg)	Drenth <i>et al.</i> , <i>J.Mol.Biol.</i> 28: 543-544 (1967) Wright <i>et al.</i> , <i>Nature</i> 221: 235-242 (1969) Petsko <i>et al.</i> , <i>J.Mol.Biol.</i> 106: 453-456 (1976)

Dear [Executor of Dr. Harrington's estate]:

I am pleased to inform you that the Congregation of Peachtree Presbyterian Church has approved a complete assignment to the Estate of Dr. W. Frank Harrington of the Church's copyright interests in Dr. Harrington's works. This assignment applies to any and all copyrights associated with Dr. Harrington's works, including derivative works, regardless of the medium in which such works were or are expressed, and regardless of how the Church acquired its interests. A copy of the minutes from the congregational meeting at which this assignment was approved is attached hereto. Please accept this letter as the assignment authorized by the congregation.

In order to make this assignment as definite as possible, we identify below some of Dr. Harrington's works that are eligible for copyright protection. However, this assignment is not limited to the interests that we identify specifically. Rather, the Church assigns to the Estate of Dr. Harrington any and all copyright interest it has or might have relating to any of Dr. Harrington's works. In effect, this assignment is a "quitclaim deed."

Written Publications: Dr. Harrington was a respected author and published many books and articles over the years. He also published many of his sermons into books and handouts. The Church does not believe that it has any interest in Dr. Harrington's written publications. However, to the extent that the Church might have such an interest it hereby assigns that interest to the Estate of Dr. Harrington.

Audio and Video Recordings: Dr. Harrington gave numerous sermons and other presentations over the years that were recorded onto audio and visual media. The Church regularly taped his sermons for television. In addition, Dr. Harrington often appeared on television and radio shows. To the extent that the Church has a copyright interest in such recordings, it hereby assigns its copyright interest in those recordings to the Estate.

Dr. Harrington's Sermons and Benediction: We understand that the underlying presentations by Dr. Harrington during Church services, including his sermons and benediction, may also be protected by copyright. To the extent that the Church has any copyright interest in such underlying presentations, it hereby assigns that interest to the Estate.

The Church is in possession of a number of items protected by these copyrights, including videos of his sermons and copies of his sermons that were distributed to the congregation on Sundays. The Church would like to retain those copies for its archives, if that is acceptable to the Estate.

We anticipate that the Church will receive occasional requests for copies of Dr. Harrington's sermons and other works. We have some ideas about how to handle those requests, and would like to discuss those ideas at your convenience.

Please let me know if you have any questions about this matter.

Peachtree Presbyterian Church

By: _____

Name:

Title: